Available online at www.sciencedirect.com
SCIENCE DIRECT®
Vaccine 21 (2003) 3854–3862
Vaccine
www.elsevier.com/locate/vaccine

Genetically engineered, live attenuated vaccines for Venezuelan equine encephalitis: testing in animal models☆

William D. Pratt[a,*], Nancy L. Davis[b], Robert E. Johnston[b], Jonathan F. Smith[c]

[a] *Virology Division, United States Army Medical Research Institute of Infectious Diseases, Fort Detrick, Frederick, MD 21702-5011, USA*
[b] *Department of Microbiology and Immunology, School of Medicine, University of North Carolina, Chapel Hill, NC 27599-7290, USA*
[c] *AlphaVax, Inc., Research Triangle Park, NC 27709-0307, USA*

Received 16 December 2002; received in revised form 31 March 2003; accepted 10 April 2003

Abstract

The central objective of this research was to test molecularly defined, live attenuated Venezuelan equine encephalitis virus (VEEV) vaccine candidates that were produced through precise genetic manipulation of rationally selected viral nucleotide sequences. Molecular clones of vaccine candidates were constructed by inserting either three independently attenuating mutations or a PE2 cleavage-signal mutation with a second-site resuscitating mutation into full-length cDNA clones. Vaccine candidate viruses were recovered through DNA transcription and RNA transfection of cultured cells, and assessed in rodent and non-human primate models. Based on results from this assessment, one of the PE2 cleavage-signal mutants, V3526, was determined to be the best vaccine candidate for further evaluation for human use.
Published by Elsevier Science Ltd.

Keywords: Venezuelan equine encephalitis virus; Vaccine; Aerosol exposure

1. Introduction

Infection with Venezuelan equine encephalitis virus (VEEV) usually results in an acute, incapacitating, but self-limiting, febrile disease in adult humans [1]. However, severe neurological infection, including fatal encephalitis, is common in infections of rodents [2,3] and horses [4], and can occur in humans, typically children [5]. Although natural disease is usually acquired through mosquito bite, VEEV is highly infectious as an aerosol and has caused many laboratory infections by this route [6]. There are two Investigational New Drug VEEV vaccines (one attenuated [TC-83], the other a killed virus vaccine [C-84]) currently used to protect laboratory workers and other at-risk personnel against VEEV [7,8], however, each has deficiencies that necessitate the development of an improved vaccine for general use [9,10].

Historically, the most effective vaccines against viral diseases have been live attenuated versions of the more virulent virus. However, the classical development of safe and effective live vaccines requires either the chance isolation of naturally attenuated viruses from nature, or the empirical development of attenuated viruses by evolving random mutations in the virulent population through, for example, passage in cell culture. In many such attempts, satisfactory live attenuated vaccines were difficult to obtain because viruses that replicated well enough to evoke protective immunity frequently retained a propensity to cause disease or revert to virulence. The contemporary tools of molecular biology now provide new routes toward rational vaccine design and construction, making it theoretically possible to engineer defined attenuating mutations into viruses to ensure an exceedingly low probability of reversion. However, even with these capabilities, the obstacles are not trivial: attenuating mutations must be identified and validated through a multidisciplinary effort in molecular virology, viral biology, viral pathogenesis, and viral immunology.

Fortunately for those efforts aimed at live attenuated vaccine development, VEEV is among the RNA viruses that can be rationally engineered by using current recombinant

☆ Research was conducted in compliance with the Animal Welfare Act and other Federal statutes and regulations relating to animals and experiments involving animals and adheres to principles stated in the Guide for the Care and Use of Laboratory Animals, National Research Council, 1996. The facility where this research was conducted is fully accredited by the Association for Assessment and Accreditation Laboratory Animal Care International.

* Corresponding author. Tel.: +1-301-619-7241; fax: +1-301-619-2290.
E-mail address: william.pratt@det.amedd.army.mil (W.D. Pratt).

0264-410X/$ – see front matter. Published by Elsevier Science Ltd.
doi:10.1016/S0264-410X(03)00328-1

Report Documentation Page

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
10 APR 2003	**N/A**	**-**

4. TITLE AND SUBTITLE	5a. CONTRACT NUMBER
Genetically engineered, live attenuated vaccines for Venezuelan equine encephalitis: testing in animal models, Vaccine 21:3854 - 3862	5b. GRANT NUMBER
	5c. PROGRAM ELEMENT NUMBER
6. AUTHOR(S)	5d. PROJECT NUMBER
Pratt, WD Davis, NL Johnston, RE Smith, JF	5e. TASK NUMBER
	5f. WORK UNIT NUMBER
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)	8. PERFORMING ORGANIZATION REPORT NUMBER
United States Army Medical Research Institute of Infectious Diseases, Fort Detrick, MD	
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)	10. SPONSOR/MONITOR'S ACRONYM(S)
	11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION/AVAILABILITY STATEMENT

Approved for public release, distribution unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

The central objective of this research was to test molecularly defined, live attenuated Venezuelan equine encephalitis virus (VEEV) vaccine candidates that were produced through precise genetic manipulation of rationally selected viral nucleotide sequences. Molecular clones of vaccine candidates were constructed by inserting either three indelendently attenuating mutations or a PE2 cleavage-signal mutation with a second-site resuscitating mutation into full-length cDNA clones. Vaccine candidate viruses were recovered through DNA transcription and RNA transfection of cultured cells, and assessed in rodent and non-human primate models. Based on results from this assessment, one of the PE2 cleavage-signal mutants, V3526, was determined to be the best vaccine candidate for further evaluation for human use.

15. SUBJECT TERMS

Venezuelan equine encephalitis virus, VEE, vaccine, aerosol exposure, laboratory animals, rodents, nonhuman primates

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			
unclassified	**unclassified**	**unclassified**	**SAR**	**9**	

DNA techniques [11]. Furthermore, there are several logical bases for choosing specific mutations to engineer into the VEE genome [12], and an array of animal models [9,13,14] with which to test the biological consequences (e.g. attenuation, immunogenicity) of such mutations. Attenuating mutations in VEE were identified by three general approaches. The first approach produced E2 mutations by passage of wild-type VEE virus in cell culture under stringent selection pressure for rapid penetration of cultured cells [15]. This approach was based on empirical observations made originally with Sindbis virus [16]. The presumed mechanism of attenuation is that changes in the surface glycoproteins that allow accelerated penetration in cultured cells result in less efficient replication in vivo, or conceivably, in different tissue tropisms. The rapid penetration mutations were defined at the nucleotide level by molecular sequencing, and reproduced individually by site-directed mutagenesis of the full-length cDNA (e.g. a Lys for Glu substitution at E2 76; a Lys for Glu substitution at E2 codon 209) [11]. The second approach to identify attenuating mutations targeted nucleotide sequences thought to be critical for optimal viral replication. The E1 81 mutant resulted from a single nucleotide substitution (a Ile for Phe) in the region of the E1 protein thought to fuse with endosomal cell membranes in the initial events of infection. The E3 Δ(56–59) and E3 59 mutants resulted from either deletion of E3 codons 56–59, or amino acid substitution of a Glu for Arg in E3 59 within the furin-like cleavage signal of PE2, the immediate precursor to mature E2 glycoprotein [17]. The PE2 cleavage-signal mutations proved to be lethal. However, after transfection with RNA from molecular clones with the PE2 cleavage-signal mutations, infectious virus was eventually recovered that contained, in addition to the original mutation, a second-site mutation at either E1 253 or E2 243, that resulted in viable virus—in effect, suppressing the lethal phenotype of the cleavage-signal mutant. These second-site mutations also resuscitated the E3 59 mutation. The third approach used a mutation, a single change (G–A) at nucleotide 3 of the 5′ untranslated region of the genome (nt3A), identified in the live attenuated IND vaccine, TC-83 [18]. Additionally, an adventitious mutation occurred at E1 codon 272 (a Thr for Ala substitution) during the site-directed mutagenesis process, which was subsequently found to be attenuating [19].

Given the high rate of mutation observed in the replication of RNA viruses, it would be expected that adequate attenuation and genetic stability of live attenuated vaccine candidates would require multiple, independently attenuating, mutations. In fact, experience with live poliovirus vaccines demonstrates that the potential for reversion to a wild-type phenotype is higher in those vaccine strains with only one or two attenuating mutations while being much less likely in those that contain more attenuating mutations [20]. Although VEEV clones with single mutations have not shown a propensity for reversion back to wild-type phenotype, clones with multiple mutations are more attenuated in sensitive animal models [17,19,21]. Infection with these mutants caused significantly less mortality in both infant mice and adult hamsters than did infection with any of the viral strains containing single mutations. Thus, inherent in the design of an appropriate vaccine candidate is the selection of the best permutation of individual mutations that achieves the desired level of attenuation and a level of genetic stability to prevent the theoretical possibility of reversion.

It is also essential that the combination of attenuating mutations chosen does not hinder the vaccine virus from inducing the appropriate effector mechanisms and immune responses sufficient to protect from naturally occurring strains. Any combination of mutations that results in low replication rates or viability in the vaccinated host may not produce the quantity of antigen or degree of host responses necessary for protective immunity. Also, combinations that directly or allosterically alter domains on the viral proteins responsible for inducing neutralizing or otherwise protective responses may result in ineffective vaccination against wild-type viruses. In this study, we examined eight potential VEEV vaccine candidates in rodent animal models. Four of these candidates were constructed with different combinations of three attenuating mutations and are referred to as triple mutants. The remaining four candidates were PE2 cleavage-signal mutants with the addition of a second-site resuscitating mutation. The three best vaccine candidates were further tested in a rigorous non-human primate model specifically designed to distinguish the most attractive candidate and to compare the immunogenicity and level of protection achieved to that achieved with the existing TC-83 vaccine and to prior infection with the wild-type virus itself.

2. Material and methods

2.1. Clones, viruses, and cells

The generation of isogenic molecular clones and viral stocks for this study was previously described. Briefly, a full-length cDNA clone of the wild-type Trinidad donkey strain of VEE (TrD), pV3000 [11], served as the template for the production of the single mutation viruses and of the multiple mutation viruses (Table 1). VEEV clones with either single or multiple mutations were constructed by using a modification of the Kunkel method [22] for site-directed mutagenesis of a M13 subclone of the glycoprotein genes in pV3000. Infectious VEEV RNA, transcribed in vitro from these clones (e.g. pV3519), was used to produce virus (e.g. termed V3519) by transfection of baby hamster kidney (BHK) cells [21]. Virus stocks tested in these studies were obtained directly from transfected BHK culture supernatant fluids and were used after appropriate dilution without further passage. Parent V3000 virus was passaged twice in BHK cells after collection from transfection supernatant fluids.

Table 1
Characteristics of mutations in attenuated mutants of VEEV

Class of mutant	Virus strain	Mutation loci	Types of mutation
Wild-type	V3000[a]	None	None: parental genotype
Single	V3010	E2 76	Single substitution (Lys for Glu) at E2 codon 76
	V3032	E2 209	Single substitution (Lys for Glu) at E2 codon 209
	V3034	E1 272	Single substitution (Thr for Ala) at E1 codon 272
	V3040	E1 253	Single substitution (Ser for Phe) at E1 codon 253
	V3042	E1 81	Single substitution (Ile for Phe) at E1 codon 81
	V3043	nt3A	Single nucleotide substitution (A for G) in the 5′ untranslated region
Triple mutants	V3519	E1 272, E2 76, E2 209	Three glycoprotein mutations
	V3520	E1 81, E2 76, E2 209	Three glycoprotein mutations
	V3522	nt3A, E2 76, E2 209	5′ Untranslated region, two glycoprotein mutations
	V3524[b]	nt3A, E1 272, E2 209	5′ Untranslated region, two glycoprotein mutations
Cleavage site mutants	V3526	E3 Δ(56–59), E1 253	Cleavage-signal deletion with resuscitating mutation
	V3528	E3 59, E1 253	Cleavage-signal mutation with resuscitating mutation
	V3531[b]	E3 Δ(56–59), E2 243	Cleavage-signal deletion with resuscitating mutation
	V3532[b]	E3 59, E2 243	Cleavage-signal mutation with resuscitating mutation

[a] The genome of V3000 differs from the Trinidad Donkey strain sequence [12] by a silent E2 170 change and an adventitious change at E2 239 (Ile for Asn). Ile at E2 239 was also identified in the sequence of a clonal isolate of the virulent Trinidad donkey strain of VEE [19] and therefore is not considered to be a candidate attenuating mutation.

[b] V3524, V3531 and V3532 contain the non-attenuating codon change (Asn for Ile) in E2 239 relative to the V3000 sequence.

2.2. Assays for virulence and immunity in rodents

Female C57BL/6 mice (8–10 weeks) and female Syrian hamsters (7–9 weeks) were inoculated s.c. with 0.2 ml of cell culture medium containing either no virus, or a calculated dose of 5×10^4 plaque forming units (pfu) of the virulent V3000 virus or one of the mutant viral strains. Groups of animals inoculated with either a single human dose (0.5 ml total dose; 0.1–0.2 ml s.c. in multiple sites) of TC-83 (National Drug Co., Philadelphia, PA, Lot 4 Run 2) or three human doses (0.5 ml total dose; 0.1–0.2 ml s.c. in multiple sites) of C-84 (The Salk Institute, Swiftwater, PA, Lot C-84-6 Run 1) on days 0, 7 and 28 were used as comparisons. The degree of attenuation of the viral strains was assessed during the 14-day observation period after inoculation. On day 49 after inoculation, surviving hamsters were bled by cardiac puncture under tiletamine-zolazepam (50 mg/kg, Aveco Co., Inc., Fort Dodge, IA) anesthesia and surviving mice were bled from the retro-orbital sinus under methoxyflurane (Pitman-Moore, Mundelein, IL) anesthesia. Serum virus-neutralizing (N) antibody titers were expressed as the final dilution neutralizing 80% of V3000 virus in a standard plaque-reduction assay (PRNT) [23]. On day 55 after the primary inoculation, animals were challenged with a calculated dose of 10^5 pfu of V3000 or 10^4 pfu of TrD by aerosol exposure or by intraperitoneal inoculation. For aerosol challenge, animals were exposed for 10 min to an infectious aerosol generated by a Collison nebulizer within a Plexiglass chamber contained within a Class III biological safety cabinet located in a Biosafety Level 3 laboratory. Viral doses delivered by aerosol were calculated by standard procedures [9]. Protection was assessed by monitoring animals for 28 days post-infection. Additional groups of animals were inoculated with selected viruses (V3526 or V3528) or TC-83 by aerosol and then challenge by aerosol with V3000 on day 55 post-inoculation to evaluate the ability of these viruses to induce mucosal immunity and protect against aerosol challenge.

2.3. Testing vaccine candidates in non-human primate model

The non-human primate model monkey used to test the safety and efficacy of VEEV vaccines was previously described [14]. Briefly, 30 healthy cynomolgus macaques (Macaca fascicularis, 4.2–6.7 kg), screened negative by ELISA for previous exposure to alphaviruses, were s.c. implanted with radiotelemetry devices (TA10TA-D70, Data Sciences, St. Paul, MN) to monitor body temperatures. During the pre-vaccination and pre-challenge periods (day −10 to day 0) and the 21 days after vaccination and challenge, body temperatures were recorded every 15 min using the PhysioTel telemetry system (Data Sciences). An autoregressive integrated moving average model [24] for each monkey was developed using the averaged hourly body temperature data over a baseline-training period (day −10 to day −3) and was used to forecast normal body temperature values during the vaccination and challenge time periods. Significant temperature elevations, represented by temperature data outside +3S.D. of the forecast values, were used to compute fever duration (number of hours or days of significant temperature elevation) and fever-hours (sum of the significant temperature elevations).

Monkeys were randomly divided into six groups ($N = 5$) and each monkey received a single s.c. 0.5 ml dose of a vaccine candidate (V3524, V3526 or V3528), TC-83,

V3000 or virus-free cell culture medium. The projected viral dosage was 5.0×10^4 pfu. On day 35 or 36 after inoculation, bronchial lavage and blood samples were collected for N antibody titrations. On days 42 or 43, monkeys were anaesthetized with tiletamine-zolazepam (3–4 mg kg^{-1}, i.m., Aveco Co, Fort Dodge, IA) and exposed for 10 min to an infectious aerosol of V3000. The average viral dosage delivered by aerosol was calculated by standard procedures [9] to be 4×10^8 pfu of V3000 or 300 ED_{50} (median effective dose) [14]. Monkeys were bled daily for 6 days after both immunization and challenge to monitor viremias and lymphocyte counts. On day 14 post-challenge, serum was collected for N antibody titrations. Virus dosages, viremias, and N antibody titers were determined in similar manner to those for the rodent studies. Statistical evaluation of the groups of monkeys was made using analysis of variance followed by multiple comparisons using the Tukey studentized range test (SAS ver. 6.10, Cary, NC).

3. Results

3.1. Evaluation of VEEV with single mutations for attenuation and protective immunity

VEEV mutants produced from full-length DNA clones with a single mutation were tested in mice and hamsters to determine their relative degree of attenuation and ability to protect from an aerosol of VEEV. All single mutants tested in mice were fully attenuated and induced protective immune responses (data not shown). These single mutants, when tested in hamsters, ranged from fully virulent to partially attenuated, with the E2 76 mutant the most attenuated single mutant tested (Table 2). The hamsters that succumbed to inoculation with a single mutant appeared to show similar signs of infection to those seen in the hamsters inoculated with wild-type virus, which is characterized by an initial lymphotropic/myelotropic stage of VEEV infection followed by bacterial overgrowth in the gut, bacteremia, and endotoxic shock with no signs of central nervous system involvement [25]. There was some delay in mean day to death with three of the single mutants, V3010, V3032, and V3042, that indicated partial attenuation. In general, hamsters that survived did generate protective immunity against aerosol challenge.

3.2. Evaluation of VEEV containing of multiple mutations in rodent animal models

A total of four triple mutant and four cleavage-signal mutant viruses were tested for safety, immunogenicity, and efficacy in both hamsters (Table 2) and mice (Table 3). TC-83 and C-84 were used as a comparison. The multiple mutants were completely avirulent in mice and most were fully attenuated in hamsters. Only V3520, V3524 and V3532 caused any death in the hamster groups, and this was only at a 5% level. In comparison, infection with TC-83 caused lethal infections in 25% of the hamsters. The multiple mutants demonstrated a high degree of variability in their potential to induce serum N antibody responses and to protect against either parenteral or aerosol VEEV challenge (Tables 2 and

Table 2
Virulence, immunology, and efficacy of molecularly cloned mutant VEEV containing single and multiple mutations in hamsters

Virus strain	S/T[a]	Serum antibody[b]		Challenge[c]	
		AB	GMT	Parenteral	Aerosol
V300	0/10				
V3010	7/10				6/7
V3032	2/10				2/2
V3034	2/10				2/2
V3040	0/10				
V3042	2/10				2/2
V3043	0/10				
V3519	20/20	6/20	+	4/10	0/10
V3520	19/20	11/18	++	4/7	3/10
V3522	20/20	17/20	++	9/9	4/10
V3524	19/20	19/19	+++	9/9	9/10
V3526	20/20	20/20	+++	9/9	0/10
V3528	20/20	18/20	++	8/10	0/10
V3531	20/20	20/20	+	8/8	1/10
V3532	19/20	14/14	+	4/4	0/10
TC-83	15/20	15/15	+++	5/5	10/10
C-84	20/20	20/20	++	5/6	0/10
Mock[i]	20/20	0/20	–	0/10	0/10

[a] S/T, number of survivors/number inoculated.
[b] Serum antibody: AB, number of animals whose serum at 1:20 dilution neutralized 80% of wild-type VEEV plaques/group total; GMT, geometric mean titer of animals with N antibody titers ≥20 ((+) 20 ≥ GMT < 100; (++) 100 ≤ GMT < 1000; (+++) 1000 ≤ GMT).
[c] Challenge, number of survivors/number challenged either by parenteral (i.p.) or aerosol VEEV.

Table 3
Virulence, immunology, and efficacy of molecularly cloned mutant VEEV containing single and multiple mutations in mice

Virus strain	S/T[a]	Serum antibody[b]		Challenge[c]	
		AB	GMT	Parenteral	Aerosol
V3519	20/20	6/20	+	6/10	2/10
V3520	20/20	8/19	+	3/9	9/10
V3522	20/20	7/20	+	5/10	3/10
V3524	20/20	20/20	+	10/10	10/10
V3526	40/40	40/40	+++	20/20	20/20
V3528	40/40	40/40	+++	20/20	20/20
V3531	20/20	20/20	++	10/10	9/10
V3532	20/20	20/20	++	10/10	10/10
TC-83	30/20	28/30	+++	14/15	14/15
C-84	20/20	20/20	++	10/10	7/10
Mock[i]	20/20	0/20	–	0/10	0/10

[a] S/T, number of survivors/number inoculated.
[b] Serum antibody: AB, number of animals whose serum at 1:20 dilution neutralized 80% of wild-type VEEV plaques/group total; GMT, geometric mean titer of animals with N antibody titers ≥20 ((+) 20 ≤ GMT < 100; (++) 100 ≤ GMT < 1000; (+++) 1000 ≤ GMT).
[c] Challenge, number of survivors/number challenged either by parenteral (i.p.) or aerosol VEEV.

3). V3519, with mutations at E2 76, E2 209 and E1 272, elicited poor N antibody responses, marginally protected animals from parenteral challenge, and provided poor protection against aerosol challenge. V3520 (E2 76, E2 209 and E1 81 mutations) and V3522 (E2 76, E2 209 and nt3A mutations) were slightly more immunogenic, but still did not fully protect all groups of hamsters and mice against either aerosol or parenteral VEEV challenge. In contrast, V3524 (E2 209, E1 272 and nt3A mutations) induced high N antibody responses in hamsters and protected most animals (90%) from aerosol challenge and all of those challenged parenterally. Interestingly, this is the only triple mutant without the E2 76 mutation, the mutation most attenuated in hamsters. In general, the serum N antibody responses of mice vaccinated with the cleavage-signal mutants were higher than those induced by the triple mutants (Table 3). V3526 and V3528 containing the E1 253 second-site suppressor mutation induced higher N antibody responses than V3531 and V3532, which contain the E2 243 second-site suppressor mutation. Protection conferred by the cleavage-signal mutants was similar; inoculated hamsters (Table 2) and mice were protected from lethal parenteral challenge, however, only mice were protected completely from aerosol challenge (Table 3). This lack of protection from aerosol VEEV challenge could be overcome by vaccinating by the aerosol route; hamsters given either V3526 or V3528 by aerosol were completely protected from a later aerosol challenge from TrD (data not shown).

3.3. Response to vaccination in a non-human primate model

V3524, V3526, and V3528 viruses were selected as vaccine candidates for further testing in cynomolgus monkeys (Table 4). As controls and comparisons in this study, separate groups of monkeys were inoculated s.c. with V3000, TC-83, or virus-free cell culture medium. Although, monkeys were inoculated with a projected dose of 5×10^4 pfu, back-titration of the innoculum indicated a range of dosages: V3524, 3.3×10^4 pfu; V3526, 4.5×10^5 pfu; V3528, 2.5×10^4 pfu; and V3000, 1.4×10^4 pfu. The average fever responses of V3524-, V3526-, V3528-, and TC-83-inoculated groups of monkeys were similar in comparison with the mock-inoculated group, with few signs of significant temperature elevation. In contrast, V3000 produced an average of 4 days of significant fever ($\geq$12 h of fever per day) in the monkeys ($P \leq 0.05$), and the four monkeys with the more severe fever responses also were viremic. Interestingly, one of the TC-83-inoculated monkeys also showed fever responses, though, not as severe as those seen in the V3000-inoculated animals. In addition, two other monkeys inoculated with TC-83 developed viremia. Viremia during the inoculation period was not detected in any of the monkeys inoculated with any of the vaccine candidates, or in the mock controls. Peripheral lymphocyte counts in the monkeys were monitored for 6 days post-inoculation and the average percent change from baseline values was compared between groups. Data from monkeys inoculated with V3000 or V3526 were significantly different from those of the mock controls and from those monkeys inoculated with V3524 ($P \leq 0.05$). Data from monkeys inoculated with either V3528 or TC-83 were not significantly different from data obtained from any of the other groups of monkeys. In all virus-inoculated groups, lymphocyte counts were back to, or above pre-inoculation baseline values by day 14, with V3000 inducing the highest increase. Monkeys inoculated with V3526 had essentially returned to baseline lymphocyte count values.

Serum and bronchial N antibody responses were similar among the V3524-, V3526-, V3528- and TC-83-inoculated groups of monkeys (Table 4) with the exception that one of the animals receiving TC-83 did not produce serum N antibody titers. Serum and bronchial N antibody responses in those monkeys receiving V3000, although statistically similar to those produced by the vaccine candidates and TC-83, suggested a more robust immune response. As

Table 4
Responses of cynomolgus monkeys immunized with live attenuated VEEV vaccine candidates

Vaccine group ($n = 5$)	Fever responses[a]				Viremia	Lymphocyte responses[b]		Serum antibody[c]		Bronchial lavage[d]
	T_{max} (°C)	Duration (days)	Duration (h)	Fever-hours (°C h)		%Decrease	%Increase	AB	GMT	
V3524	1.2	0.2 ± 0.4	11.4	10.0	0/5	−18	6	5/5	735	2/5
V3526	1.6	0.2 ± 0.4	19	18.3	0/5	−27	2	5/5	2229	3/5
V3528	1.7	0.0	20	18.8	0/5	−13	16	5/5	3880	2/5
TC-83	1.4	0.4 ± 0.9	32.8	28.2	2/5	−17	10	4/5	905	3/5
V3000	2.6	4.0 ± 3.2	101.2	136.5	4/5	−35	43	5/5	8914	4/5
Mock	1.3	0.2 ± 0.4	37	31.3	0/5	−9	3	0/5	<20	0/5

[a] Fever responses: the group means of the maximum temperature elevation (T_{max}); of the number of days (duration [days]) or hours (duration [hours]) monkeys displayed significant temperature elevation; and of the sum of the temperature elevations (fever-hours).

[b] Peripheral blood lymphocyte responses were examined by evaluating the following parameters: %decrease, the group mean of the average percent decrease in blood lymphocyte counts from baseline (day −2 through day 0) during the 6 days post-inoculation; %increase, the group mean of the average percent increase in blood lymphocyte counts from baseline at day 14 post-inoculation.

[c] Serum antibody: AB, number of animals at day 45 after immunization whose serum at 1:20 dilution neutralized ≥80% of VEEV plaques in a PRNT/group total; GMT, geometric mean titer of animals with N antibody titers ≥20.

[d] Bronchial lavage, number of animals at day 45 after immunization whose bronchial lavage at 1:2 dilution neutralized ≥80% of VEEV plaques.

Table 5
Responses of cynomolgus monkeys challenged with wild-type VEEV (V3000) by the aerosol route

Vaccine group ($n = 5$)	Fever responses				Viremia	Lymphocyte responses		PRNT increase[a]
	T_{max} (°C)	Duration (days)	Duration (h)	Fever-hours (°C h)		%Decrease	%Increase	
V3524	2.9	3.8 ± 1.8	84.8	159.7	1/5	−22	28	574
V3526	2.2	1.2 ± 1.1	37.7	54.6	0/5	−17	4	7
V3528	2.6	2.0 ± 2.1	49.4	80.1	0/5	−6	13	7
TC-83	2.8	2.8 ± 2.2	66.2	113.1	0/5	−15	27	36
V3000	1.7	1.4 ± 2.1	34.0	59.7	0/5	−9	19	4
Mock	3.7	6.8 ± 0.8	154.2	338.4	5/5	−52	33	1024

[a] PRNT increase (immunostimulation), the ratio of the group mean serum N antibody titers of post-challenge sera (day 14) to the pre-challenge sera (day −1) in VEEV neutralization in vitro as measured by PRNT. N antibody titers of <20 were calculated as 10.

expected, none of the mock control monkeys showed any VEEV-specific immune responses to mock inoculation.

3.4. Response to challenge in the non-human primate model

Typical fever responses to aerosol infection with V3000 in the cynomolgus monkey model with radiotelemetry devices were previously shown [14], and very similar responses were seen in this study (Table 5). Fever responses were usually seen by the second 24 h period after aerosol challenge and were present for 6–7 days. Viremia and lymphopenia were also observed within this time period. In this study, one mock-inoculated monkey developed signs of encephalitis, became hypothermic, and was humanely killed. Pathology of this animal showed panencephalitis in areas in the cerebral cortex, substantia nigra, and hypothalamus.

Fever responses in the inoculated monkeys varied from signs similar to those seen in mock-inoculated monkeys to signs of a low-level fever only during the second to fourth day after challenge, to the absence of signs of fever. As groups, the vaccinated monkeys could be placed into two distinct categories. Those groups of monkeys vaccinated with V3526 or V3528 were well protected against aerosol challenge with few to no signs of fever, lymphopenia, or viremia—similar to the group of monkeys previously inoculated with V3000. The group of monkeys vaccinated with TC-83 was also in this category, but it is noteworthy that the one monkey that did not have pre-challenge N antibody titers did develop fever responses similar to the mock-inoculated monkeys. Unlike the groups of monkeys inoculated with V3526, V3528, TC-83, or V3000, the group of monkeys inoculated with V3524 was not as well protected against aerosol challenge with V3000 and was in a distinct fever grouping. These animals showed significantly higher fever responses compared to the other candidate-vaccinated groups ($P \leq 0.05$), and a significantly higher degree of lymphopenia compared to the V3000 previously inoculated group ($P \leq 0.05$). The V3524-inoculated group of monkeys also demonstrated a high degree of immunostimulation from the challenge as measured by the increase in serum N antibody titers after challenge, suggesting a higher degree of infection and viral replication in these monkeys. Additionally, viremia was present in one V3524-inoculated monkey.

The serum N antibody titers of all monkeys at 45 days post-inoculation were examined relative to signs of disease to determine if this value correlated with protection from aerosol challenge, and thus could be used as a surrogate marker for future vaccine trials in humans (Fig. 1). We found that serum N antibody titers ($\log_{10}$) negatively correlated with the fever responses (Pearson correlation coefficients: duration, $r = -0.89$, $P = 0.0001$ (Fig. 1); fever-hours, $r = -0.86$, $P = 0.0001$), and with lymphopenia ($r = -0.77$, $P = 0.0001$). These correlations are quite good for biological endpoints and indicate that 60% of the variation in lymphopenia and almost 80% of the variation in duration and fever-hours can be accounted for by linear association with serum N antibody titers.

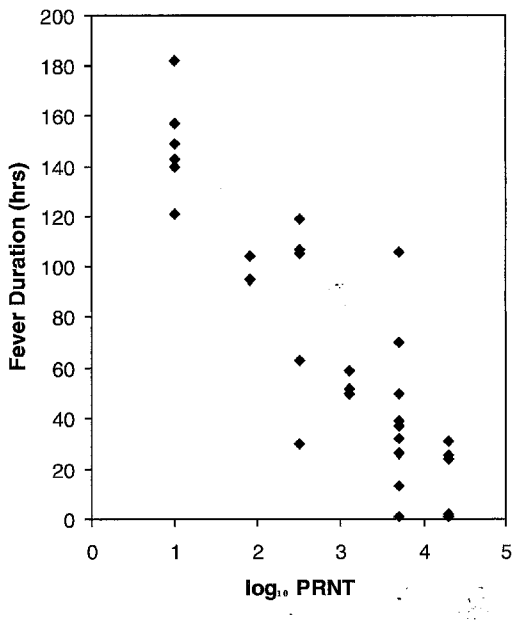

Fig. 1. Correlation between serum N antibody titers and fever duration after aerosol challenge with virulent VEEV in monkeys ($n = 30$). Serum N antibody titers ($\log_{10}$) from individual monkeys (◆) negatively correlated with their duration (h) of significant fever responses ($r = -0.89$, $P = 0.0001$). N antibody titers of <20 were calculated as 10.

4. Discussion

In this study, we successfully tested and expanded on the hypothesis that safe and effective, live attenuated vaccines for VEEV can be rationally designed and constructed using current genetic engineering technologies. The phenotypic properties of the viruses generated from clones in this study and in past studies [17,19,21,26–30] provide the formal evidence that the identified single mutations were responsible for the observed attenuation. From the list of mutations, vaccine candidates for VEEV were constructed to provide a higher level of attenuation and a mathematical improbability of reversion to wild-type phenotype after production and vaccination. Overall, this approach appeared to be remarkably successful insofar as five out of eight vaccine candidates evaluated were fully attenuated in both rodent species. Only V3520, V3524, and V3532 produce disease and death in hamsters, but did so at a much lower level than the single mutants or the current IND vaccine, TC-83. Three candidates, V3524, V3526, and V3528, were highly immunogenic and provided excellent protection in mice against not only parenteral challenge, but also against the more rigorous aerosol challenge. However, only V3524 and TC-83 protected hamsters against an aerosol challenge, and clearly, this is the most vigorous of the rodent models used in this study. V3524 is the only triple mutant without the E2 76 mutation, the most attenuating mutation tested in hamsters. It appears that the E2 76 mutation, in combination with other mutations, results in over-attenuated viruses that cannot consistently induce protective immune responses. The lack of aerosol protection in hamsters could be overcome by changing the route of vaccination; animals vaccinated by an aerosol of either V3526 or V3528 were completely protected. This study, and previous aerosol challenge studies in hamsters [13] or in C3H mice [28], strongly suggest that a specific immune response, probably mucosal, is required for protection in these vigorous rodent challenge models, and that the detection of serum N antibody alone is not necessarily predictive of protection. Giving V3526 or V3528 by aerosol most probably induced mucosal responses in these hamsters of sufficient magnitude to protect against aerosol challenge. The study by Hart et al. [28] in C3H/HeN mice supports this presumption. They found that V3526 and TC-83 given by the s.c. route could not protect this strain of mice from TrD aerosol challenge, whereas V3526 given by aerosol could, and that this was associated with more consistent and higher antiviral IgA responses in the nasal and bronchial washes of vaccination-matched groups of mice.

The most promising candidates, V3524, V3526, and V3528, were further tested in a cynomolgus monkey model which uses an extremely high challenge dose of wild-type virus by aerosol [14]. We found all vaccine candidates to be safe, whereas, V3000 induced significant fever, viremia, and lymphopenia. Similar responses were seen in one TC-83-inoculated monkey, but at a lower level. This observation was not surprising since TC-83 is know to cause significant side-effects of fever, headache, and malaise in approximately 23% of vaccinated people [10], and is a main reason for the need to develop a new VEE vaccine. Furthermore, a different TC-83-inoculated monkey failed to develop detectable antibody responses. This additional limitation of TC-83 is also commonly seen in people where only 82% of the vaccinees respond with a serum N titer ($\geq$1:20). One candidate, V3526, did cause significant lymphopenia within the initial 6 days post-vaccination, but this was not associated with fever or viremia in any of the monkeys in this group and the peripheral lymphocyte levels had returned to baseline by 14 days post-vaccination. It is possible that the peripheral lymphocytes migrated into extravacular spaces in a homing response to virus-induced cytokines and not lymphocyte death. All monkeys inoculated with one of the vaccine candidates produced serum N antibodies; however, the levels seen in the two PE2 cleavage-signal mutants, V3526 and V3528, were most similar to those seen in the wild-type VEEV-inoculated group.

As would be predicted from the pre-challenge immune responses, V3526 and V3528 protected monkeys against a severe aerosol challenge of VEEV as well as V3000, inducing few signs of infection and disease, and were clearly superior to the triple mutant, V3524 and the IND vaccine, TC-83. The monkeys inoculated with V3524 were only partially protected, as demonstrated by lower grade of fever (fever-hours), fewer days of fever, and more moderate lymphocyte responses than those seen in mock-inoculated controls. V3524's failure to induce a robust protective immune response in monkeys may be due to one of its mutations—the nt3A mutation in the noncoding region of the viral genome was recently shown to increase the virus's sensitivity to interferon [31]. Interestingly, this mutation is also one of the mutations that distinguish the TC-83 vaccine strain from its virulent parent, the TrD strain, and could be the cause of TC-83's significant nonresponder rate. This lack of response to vaccination translated to lack of protection—the one monkey that did not respond to TC-83 had fever responses similar to those seen in the mock-inoculated group. Those TC-83-inoculated monkeys that did respond with serum N antibody were as protected as those inoculated with V3526 or V3528. Across groups, serum N antibody titer predicted the level of protection, with those monkeys with higher serum N antibody titers showing significantly fewer signs of fever and lymphopenia, and thus provides a correlate of protection and a possible human surrogate marker. This is an important element in the path to licensure for a VEEV vaccine for use against an aerosol delivery of the virus, as would be the case in the vaccine's use against the threat of bioterrorism. For such an application, FDA approval would need to be done under the new "Animal Efficacy Rule", and would rely on the evidence from studies such as this one to provide substantial evidence of the effectiveness of the vaccine.

Based on our results in non-human primates and those from cohort studies in rodents [17,28–30] and mosquitoes

[32], V3526 was selected for development into a human-use vaccine. These other studies, like this study, showed V3526 to be more safe, immunogenic and efficacious than TC-83, and without any indication of reversion to virulent phenotype. The primary reason for choosing V3526 over V3528 was the additional level of safety afforded by the design of this particular molecular clone. While both clones have PE2 cleavage-signal site mutations, V3526 has the full deletion of the 12 nucleotides that code for the amino acids that make up the PE2 cleavage-signal sequence, whereas V3528 has only a two-nucleotide change in E3 59 codon to provide the PE2 phenotype. Although we have seen no evidence of phenotypic reversion in V3528, we felt the additional level of safety in the design was prudent when all other factors were equal. An additional level of safety in the PE2 cleavage-signal mutants is provided by the second-site suppressor mutation at E1 253, which is attenuating on its own, but also rescues the otherwise lethal PE2 cleavage-signal mutation [17]. Reversion at the E1 253 locus results in nonviable viral progeny and regeneration of the PE2 cleavage signal would still result in attenuated progeny. An additional level of safety is incorporated in the proposed manufacturing process where DNA is used as the master seed stock and viral replication is limited to two passages in cell culture after transfection. Recent consensus sequence analysis of a manufactured pilot lot of V3526 performed by DynPort Vaccine Corporation showed no deviation from the original cDNA sequence. In summary, this study provides some of the final analysis in the development of a new VEEV vaccine for human use by means of a novel approach in vaccine development. Such an approach is highly relevant to the development of other live attenuated viral vaccines.

Acknowledgements

We thank Ralph Tammariello, Debra Kefauver, and Ryan Rostad for providing technical assistance; Catherine Wilhelmsen for the pathology; and Paul Gibbs for performing statistical analysis. Drs. Mary Kate Hart and Douglas S. Reed are gratefully acknowledged for reviewing the manuscript. The views of the authors do not purport to reflect the positions of the Department of the Army or the Department of Defense. N.L.D. and R.E.J. were supported by grant DAMD17-94-J-4430 from the US Army Research and Materiel Command. DynPort Vaccine Corporation is supported by contract DAMD17-98-C-8024 with the Joint Vaccine Acquisition Program under the Department of the Army.

References

[1] Johnson KM, Martin DH. Venezuelan equine encephalitis. Adv Vet Sci Comp Med 1974;18(0):79–116.

[2] Charles PC, Walters E, Margolis F, Johnston RE. Mechanism of neuroinvasion of Venezuelan equine encephalitis virus in the mouse. Virology 1995;208(2):662–71.

[3] Vogel P, Abplanalp D, Kell W, et al. Venezuelan equine encephalitis in BALB/c mice: kinetic analysis of central nervous system infection following aerosol or subcutaneous inoculation. Arch Pathol Lab Med 1996;120(2):164–72.

[4] Walton TE, Alvarez Jr O, Buckwalter RM, Johnson KM. Experimental infection of horses with enzootic and epizootic strains of Venezuelan equine encephalomyelitis virus. J Infect Dis 1973;128(3):271–82.

[5] Sanmartin C, Mackenzie RB, Trapido H, et al. Venezuelan equine encephalitis in Colombia, 1967. Bol Oficina Sanit Panam 1973;74(2):108–37.

[6] Health, CfDCaPNIo, editor. Biosafety in microbiological and biomedical laboratories. Washington: US Government Printing Office; 1999.

[7] Berge TO, Banks IS, Tigertt WD. Attenuation of Venezuelan equine encephalomyelitis virus in vitro cultivation in guinea-pig heart cells. Am J Hyg 1961;73:209–18.

[8] Cole Jr FE, May SW, Robinson DM. Formalin-inactivated Venezuelan equine encephalomyelitis (Trinidad strain) vaccine produced in rolling-bottle cultures of chicken embryo cells. Appl Microbiol 1973;25(2):262–5.

[9] Hart MK, Pratt WD, Panelo F, Tammariello R, Dertzbaugh M. Venezuelan equine encephalitis virus vaccines induce mucosal IgA responses and protection from airborne infection in BALB/c, but not C3H/HeN mice. Vaccine 1997;15(4):363–9.

[10] Pittman PR, Makuch RS, Mangiafico JA, Cannon TL, Gibbs PH, Peters CJ. Long-term duration of detectable neutralizing antibodies after administration of live-attenuated VEE vaccine and following booster vaccination with inactivated VEE vaccine. Vaccine 1996;14(4):337–43.

[11] Davis NL, Willis LV, Smith JF, Johnston RE. In vitro synthesis of infectious venezuelan equine encephalitis virus RNA from a cDNA clone: analysis of a viable deletion mutant. Virology 1989;171(1):189–204.

[12] Kinney RM, Johnson BJ, Welch JB, Tsuchiya KR, Trent DW. The full-length nucleotide sequences of the virulent Trinidad donkey strain of Venezuelan equine encephalitis virus and its attenuated vaccine derivative, strain TC-83. Virology 1989;170(1):19–30.

[13] Jahrling PB, Stephenson EH. Protective efficacies of live attenuated and formaldehyde-inactivated Venezuelan equine encephalitis virus vaccines against aerosol challenge in hamsters. J Clin Microbiol 1984;19(3):429–31.

[14] Pratt WD, Gibbs P, Pitt ML, Schmaljohn AL. Use of telemetry to assess vaccine-induced protection against parenteral and aerosol infections of Venezuelan equine encephalitis virus in non-human primates. Vaccine 1998;16(9–10):1056–64.

[15] Johnston RE, Smith JF. Selection for accelerated penetration in cell culture coselects for attenuated mutants of Venezuelan equine encephalitis virus. Virology 1988;162(2):437–43.

[16] Olmsted RA, BRS, Sawyer BA, Johnston RE. Sindbis virus mutants selected for rapid growth in cell culture display attenuated virulence in animals. Science 1984;225(4660):424–7.

[17] Davis NL, Brown KW, Greenwald GF, et al. Attenuated mutants of Venezuelan equine encephalitis virus containing lethal mutations in the PE2 cleavage signal combined with a second-site suppressor mutation in E1. Virology 1995;212(1):102–10.

[18] Kinney RM, Chang GJ, Tsuchiya KR, et al. Attenuation of Venezuelan equine encephalitis virus strain TC-83 is encoded by the 5′-noncoding region and the E2 envelope glycoprotein. J Virol 1993;67(3):1269–77.

[19] Grieder FB, Davis NL, Aronson JF, et al. Specific restrictions in the progression of Venezuelan equine encephalitis virus-induced disease resulting from single amino acid changes in the glycoproteins. Virology 1995;206(2):994–1006.

[20] Almond JW. The attenuation of poliovirus neurovirulence. Annu Rev Microbiol 1987;41:153–80.
[21] Davis NL, Powell N, Greenwald GF, et al. Attenuating mutations in the E2 glycoprotein gene of Venezuelan equine encephalitis virus: construction of single and multiple mutants in a full-length cDNA clone. Virology 1991;183(1):20–31.
[22] Kunkel TA. Rapid and efficient site-specific mutagenesis without phenotypic selection. Proc Natl Acad Sci USA 1985;82(2):488–92.
[23] Earley E, Peralta PH, Johnson KM. A plaque neutralization method for arboviruses. Proc Soc Exp Biol Med 1967;125:741–7.
[24] BMDP Statistical Software, Release 7; 1992. p. 467.
[25] Gorelkin L, Jahrling PB. Virus-initiated septic shock. Acute death of Venezuelan encephalitis virus-infected hamsters. Lab Invest 1975;32(1):78–85.
[26] Grieder FB, Nguyen HT. Virulent and attenuated mutant Venezuelan equine encephalitis virus show marked differences in replication in infection in murine macrophages. Microb Pathog 1996;21(2):85–95.
[27] Charles PC, Brown KW, Davis NL, Hart MK, Johnston RE. Mucosal immunity induced by parenteral immunization with a live attenuated Venezuelan equine encephalitis virus vaccine candidate. Virology 1997;228(2):153–60.
[28] Hart MK, Caswell-Stephan K, Bakken R, et al. Improved mucosal protection against Venezuelan equine encephalitis virus is induced by the molecularly defined, live-attenuated V3526 vaccine candidate. Vaccine 2000;18(26):3067–75.
[29] Steele KE, Davis KJ, Stephan K, Kell W, Vogel P, Hart MK. Comparative neurovirulence and tissue tropism of wild-type and attenuated strains of Venezuelan equine encephalitis virus administered by aerosol in C3H/HeN and BALB/c mice. Vet Pathol 1998;35(5):386–97.
[30] Ludwig GV, Turell MJ, Vogel P, et al. Comparative neurovirulence of attenuated and non-attenuated strains of Venezuelan equine encephalitis virus in mice. Am J Trop Med Hyg 2001;64(1–2): 49–55.
[31] White LJ, Wang JG, Davis NL, Johnston RE. Role of alpha/beta interferon in Venezuelan equine encephalitis virus pathogenesis: effect of an attenuating mutation in the 5′ untranslated region. J Virol 2001;75(8):3706–18.
[32] Turell MJ, Ludwig GV, Kondig J, Smith JF. Limited potential for mosquito transmission of genetically engineered, live-attenuated Venezuelan equine encephalitis virus vaccine candidates. Am J Trop Med Hyg 1999;60(6):1041–4.